PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
G01N 1/34, 33/48, C12N 5/00, 1/21, A61K 37/02, 48/00, C07K 13/00, 15/28, C07H 21/04, C12N 15/12, 15/64, A01K 67/00

A1

(11) International Publication Number: WO 94/28391

(43) International Publication Date: 8 December 1994 (08.12.94)

(21) International Application Number: PCT/US94/05365

(22) International Filing Date: 12 May 1994 (12.05.94)

(30) Priority Data:

08/068,394	24 May 1993 (24.05.93)	US
08/106,463	12 August 1993 (12.08.93)	US
08/111,758	25 August 1993 (25.08.93)	US
08/162,407	3 December 1993 (03.12.93)	US
08/209,502	7 March 1994 (07.03.94)	US

(71) Applicant: IMMUNEX CORPORATION [US/US]; 51 University Street, Seattle, WA 98101 (US).

(72) Inventors: LYMAN, Stewart, D.; 312 N. 45th Street, Seattle, WA (US). BECKMANN, M., Patricia; 5454 Ragan Lane, Poulsbo, WA 98370 (US).

(74) Agent: MALASKA, Stephen, L.; Immunex Corporation, 51 University Street, Seattle, WA 98101 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MD, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: LIGANDS FOR FLT3 RECEPTORS

(57) Abstract

Ligands for flt3 receptors capable of transducing self-renewal signals to regulate the growth, proliferation or differentiation of progenitor cells and stem cells are disclosed. The invention is directed to flt3-L as an isolated protein, the DNA encoding the flt3-L, host cells transfected with cDNAs encoding flt3-L, compositions comprising flt3-L, methods of improving gene transfer to a mammal using flt3-L, and methods of improving transplantations using flt3-L. Flt3-L finds use in treating patients with anemia, AIDS and various cancers.

TITLE

LIGANDS FOR FLT3 RECEPTORS

CROSS-REFERENCE TO RELATED APPLICATIONS

This is a continuation-in-part of United States Application 08/209,502 filed March 7, 1994, which is a continuation-in-part of United States Application 08/162,407, filed December 3, 1993, which is a continuation-in-part of United States Application 08/111,758, filed August 25, 1993, which is a continuation-in-part of United States Application 08/106,463, filed August 12, 1993, which in turn is a continuation-in-part of United States Application 08/068,394, filed May 24, 1993, abandoned.

FIELD OF THE INVENTION

The present invention relates to mammalian flt3-ligands, the nucleic acids encoding such ligands, processes for production of recombinant flt3-ligands, pharmaceutical compositions containing such ligands, and their use in various therapies.

BACKGROUND OF THE INVENTION

Blood cells originate from hematopoietic stem cells that become committed to differentiate along certain lineages, i.e., erythroid, megakaryocytic, granulocytic, monocytic, and lymphocytic. Cytokines that stimulate the proliferation and maturation of cell precursors are called colony stimulating factors ("CSFs"). Several CSFs are produced by T-lymphocytes, including interleukin-3 ("IL-3"), granulocyte-monocyte CSF (GM-CSF), granulocyte CSF (G-CSF), and monocyte CSF (M-CSF). These CSFs affect both mature cells and stem cells. Heretofore no factors have been discovered that are able to predominantly affect stem cells.

Tyrosine kinase receptors ("TKRs") are growth factor receptors that regulate the proliferation and differentiation of a number of cells (Yarden, Y. & Ullrich, A. *Annu. Rev. Biochem.*, 57, 443-478, 1988; and Cadena, D.L. & Gill, G.N. *FASEB J.*, 6, 2332-2337, 1992). Certain TKRs function within the hematopoietic system. For example, signaling through the colony-stimulating factor type 1 ("CSF-1"), receptor c-fms regulates the survival, growth and differentiation of monocytes (Stanley *et al.*, *J.*

erythropoietin ("EPO") and combinations thereof in autologous transplantation regimens.

High-dose chemotherapy is therapeutically beneficial because it can produce an increased frequency of objective response in patients with metastatic cancers, particularly breast cancer, when compared to standard dose therapy. This can result in extended disease-free remission for some even poor-prognosis patients. Nevertheless, high-dose chemotherapy is toxic and many resulting clinical complications are related to infections, bleeding disorders and other effects associated with prolonged periods of myelosuppression.

Myelodysplastic syndromes are stem cell disorders characterized by impaired cellular maturation, progressive pancytopenia, and functional abnormalities of mature cells. They have also been characterized by variable degrees of cytopenia, ineffective erythropoiesis and myelopoiesis with bone marrow cells that are normal or increased in number and that have peculiar morphology. Bennett et. al. (*Br. J. Haematol.* 1982; 51:189-199) divided these disorders into five subtypes: refractory anemia, refractory anemia with ringed sideroblasts, refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, and chronic myelomonocytic leukemia. Although a significant percentage of these patients develop acute leukemia, a majority die from infectious or hemorrhagic complications. Treatment of theses syndromes with retinoids, vitamin D, and cytarabine has not been successful. Most of the patients suffering from these syndromes are elderly and are not suitable candidates for bone marrow transplantation or aggressive antileukemic chemotherapy.

Aplastic anemia is another disease entity that is characterized by bone marrow failure and severe pancytopenia. Unlike myelodysplastic syndrome, the bone marrow is acellular or hypocellular in this disorder. Current treatments include bone marrow transplantation from a histocompatible donor or immunosuppressive treatment with antithymocyte globulin (ATG). Similarly to myelodysplastic syndrome, most patients suffering from this syndrome are elderly and are unsuitable for bone marrow transplantation or for aggressive antileukemic chemotherapy. Mortality in these patients is exceedingly high from infectious or hemorrhagic complications.

Anemia is common in patients with acquired immune deficiency syndrome (AIDS). The anemia is usually more severe in patients receiving zidovudine therapy. Many important retroviral agents, anti-infectives, and anti-neoplastics suppress

following cytoreductive therapy to counteract the myelosuppressive effects of such therapy. The present invention provides for the use of an effective amount of flt3-L in at least one of the following manners: (i) flt3-L is administered to the patient prior to collection of the progenitor or stem cells to increase or mobilize the numbers of such circulating cells; (ii) following collection of the patient's progenitor or stem cells, flt3-L is used to expand such cells *ex vivo*; and (iii) flt3-L is administered to the patient following transplantation of the collected progenitor or stem cells to facilitate engraftment thereof. The transplantation method of the invention can further comprise the use of an effective amount of a cytokine in sequential or concurrent combination with the flt3-L. Such cytokines include, but are not limited to interleukins ("IL") IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14 or IL-15, a CSF selected from the group consisting of G-CSF, GM-CSF, M-CSF, or GM-CSF/IL-3 fusions, or other growth factors such as CSF-1, SF, EPO, leukemia inhibitory factor ("LIF") or fibroblast growth factor ("FGF"). The flt3-L is also useful in the same way for syngeneic or allogeneic transplantations.

The invention further includes a progenitor or stem cell expansion media comprising cell growth media, autologous serum, and flt3-L alone or in combination with a cytokine from the group listed above.

The invention further includes the use of flt3-L to expand progenitor or stem cells collected from umbilical cord blood. The expansion may be performed with flt3-L alone or in sequential or concurrent combination with a cytokine from the group listed above.

The invention further includes the use of flt3-L in gene therapy. Flt3-L permits proliferation and culturing of the early hematopoietic progenitor or stem cells that are to be transfected with exogenous DNA for use in gene therapy. Alternatively, a cDNA encoding flt3-L may be transfected into cells in order to ultimately deliver its gene product to the targeted cell or tissue.

In addition, the invention includes the use of flt3-L to stimulate production of erythroid cells *in vivo* for the treatment of anemia. Such use comprises administering flt3-L to the patient in need of such erythroid cell stimulation in conjunction with or following cytoreductive therapy. The treatment can include co-administration of another growth factor selected from the cytokines from the group listed above. Preferred cytokines for use in this treatment include EPO, IL-3, G-CSF and GM-CSF.

finds distribution in the testis, ovaries, lymph node, spleen, thymus and fetal liver, treatment of a variety of conditions associated with tissue damage thereof is possible. While not limited to such, particular uses of the flt3-L are described infra.

As used herein, the term "flt3-L" refers to a genus of polypeptides that bind and complex independently with flt3 receptor found on progenitor and stem cells. The term "flt3-L" encompasses proteins having the amino acid sequence 1 to 231 of SEQ ID NO:2 or the amino acid sequence 1 to 235 of SEQ ID NO:6, as well as those proteins having a high degree of similarity or a high degree of identity with the amino acid sequence 1 to 231 of SEQ ID NO:2 or the amino acid sequence 1 to 235 of SEQ ID NO:6, and which proteins are biologically active and bind the flt3 receptor. In addition, the term refers to biologically active gene products of the DNA of SEQ ID NO:1 or SEQ ID NO:5. Further encompassed by the term "flt3-L" are the membrane-bound proteins (which include an intracellular region, a membrane region, and an extracellular region), and soluble or truncated proteins which comprise primarily the extracellular portion of the protein, retain biological activity and are capable of being secreted. Specific examples of such soluble proteins are those comprising the sequence of amino acids 28-163 of SEQ ID NO:2 and amino acids 28-160 of SEQ ID NO:6.

The term "biologically active" as it refers to flt3-L, means that the flt3-L is capable of binding to flt3. Alternatively, "biologically active" means the flt3-L is capable of transducing a stimulatory signal to the cell through the membrane-bound flt3.

"Isolated" means that flt3-L is free of association with other proteins or polypeptides, for example, as a purification product of recombinant host cell culture or as a purified extract.

A "flt3-L variant" as referred to herein, means a polypeptide substantially homologous to native flt3-L, but which has an amino acid sequence different from that of native flt3-L (human, murine or other mammalian species) because of one or more deletions, insertions or substitutions. The variant amino acid sequence preferably is at least 80% identical to a native flt3-L amino acid sequence, most preferably at least 90% identical. The percent identity may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the

hematopoietic cell population. Of the foregoing, SF, IL-1, IL-3, EPO, G-CSF, GM-CSF and GM-CSF/IL-3 fusions are preferred, with G-CSF, GM-CSF and GM-CSF/IL-3 fusions being especially preferred. The term "allogeneic transplantation" means a method in which bone marrow or peripheral blood progenitor cells or stem cells are removed from a mammal and administered to a different mammal of the same species. The term "syngeneic transplantation" means the bone marrow transplantation between gentically identical mammals.

The transplantation method of the invention described above optionally comprises a preliminary *in vivo* procedure comprising administering flt3-L alone or in sequential or concurrent combination with a recruitment growth factor to a patient to recruit the hematopoietic cells into peripheral blood prior to their harvest. Suitable recruitment factors are listed above, and preferred recruitment factors are flt3-L, SF, IL-1 and IL-3.

The method of the invention described above optionally comprises a subsequent *in vivo* procedure comprising administering flt3-L alone or in sequential or concurrent combination with an engraftment growth factor to a patient following transplantation of the cellular preparation to facilitate engraftment and augment proliferation of engrafted hematopoietic progenitor or stem cells from the cellular preparation. Suitable engraftment factors are listed above, and the preferred engraftment factors are GM-CSF, G-CSF, IL-3, IL-1, EPO and GM-CSF/IL-3 fusions.

The invention further includes a progenitor or stem cell expansion media comprising cell growth media, autologous serum, and flt3-L alone or in combination with a cytokine growth factor from the list above. Preferred growth factors are SF, GM-CSF, IL-3, IL-1, G-CSF, EPO, and GM-CSF/IL-3 fusions.

In particular, flt3-L can be used to stimulate the proliferation of hematopoietic and non-hematopoietic stem cells. Such stimulation is beneficial when specific tissue damage has occurred to these tissues. As such, flt3-L may be useful in treating neurological damage and may be a growth factor for nerve cells. It is probable that flt3-L would be useful in *in vitro* fertilization procedures and likely can be used *in vivo* in the treatment of infertility conditions. Flt3-L would be useful in treating intestinal damage resulting from irradiation or chemotherapy. Since the flt3 receptor is distributed on stem cells leading to the development of hair follicles, flt3-L would likely be useful to promote hair growth.

binding of flt3-L, in view of the possibility that the ligand being sought would be multimeric.

As described supra., an aspect of the invention is soluble flt3-L polypeptides. Soluble flt3-L polypeptides comprise all or part of the extracellular domain of a native flt3-L but lack the transmembrane region that would cause retention of the polypeptide on a cell membrane. Soluble flt3-L polypeptides advantageously comprise the native (or a heterologous) signal peptide when initially synthesized to promote secretion, but the signal peptide is cleaved upon secretion of flt3-L from the cell. Soluble flt3-L polypeptides encompassed by the invention retain the ability to bind the flt3 receptor. Indeed, soluble flt3-L may also include part of the transmembrane region or part of the cytoplasmic domain or other sequences, provided that the soluble flt3-L protein can be secreted.

Soluble flt3-L may be identified (and distinguished from its non-soluble membrane-bound counterparts) by separating intact cells which express the desired protein from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired protein. The presence of flt3-L in the medium indicates that the protein was secreted from the cells and thus is a soluble form of the desired protein.

Soluble forms of flt3-L possess many advantages over the native bound flt3-L protein. Purification of the proteins from recombinant host cells is feasible, since the soluble proteins are secreted from the cells. Further, soluble proteins are generally more suitable for intravenous administration.

Examples of soluble flt3-L polypeptides include those comprising a substantial portion of the extracellular domain of a native flt3-L protein. Such soluble mammalian flt3-L proteins comprise amino acids 28 through 188 of SEQ ID NO:2 or amino acids 28 through 182 of SEQ ID NO:6. In addition, truncated soluble flt3-L proteins comprising less than the entire extracellular domain are included in the invention. Such truncated soluble proteins are represented by the sequence of amino acids 28-163 of SEQ ID NO:2, and amino acids 28-160 of SEQ ID NO:6. When initially expressed within a host cell, soluble flt3-L may additionally comprise one of the heterologous signal peptides described below that is functional within the host cells employed. Alternatively, the protein may comprise the native signal peptide, such that the mammalian flt3-L comprises amino acids 1 through 188 of SEQ ID NO:2 or amino

any of a number of conventional methods. Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene wherein predetermined codons can be altered by substitution, deletion or insertion. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); Kunkel (*Proc. Natl. Acad. Sci. USA* 82:488, 1985); Kunkel et al. (*Methods in Enzymol.* 154:367, 1987); and U.S. Patent Nos. 4,518,584 and 4,737,462 all of which are incorporated by reference.

Flt3-L may be modified to create flt3-L derivatives by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of flt3-L may be prepared by linking the chemical moieties to functional groups on flt3-L amino acid side chains or at the N-terminus or C-terminus of a flt3-L polypeptide or the extracellular domain thereof. Other derivatives of flt3-L within the scope of this invention include covalent or aggregative conjugates of flt3-L or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugate may comprise a signal or leader polypeptide sequence (e.g. the α-factor leader of *Saccharomyces*) at the N-terminus of a flt3-L polypeptide. The signal or leader peptide co-translationally or post-translationally directs transfer of the conjugate from its site of synthesis to a site inside or outside of the cell membrane or cell wall.

Flt3-L polypeptide fusions can comprise peptides added to facilitate purification and identification of flt3-L. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988.

The invention further includes flt3-L polypeptides with or without associated native-pattern glycosylation. Flt3-L expressed in yeast or mammalian expression systems (e.g., COS-7 cells) may be similar to or significantly different from a native

Nucleic acid sequences within the scope of the invention include isolated DNA and RNA sequences that hybridize to the native flt3-L nucleotide sequences disclosed herein under conditions of moderate or severe stringency, and which encode biologically active flt3-L. Conditions of moderate stringency, as defined by Sambrook et al. *Molecular Cloning: A Laboratory Manual,* 2 ed. Vol. 1, pp. 1.101-104, Cold Spring Harbor Laboratory Press, (1989), include use of a prewashing solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization conditions of about 55°C, 5 X SSC, overnight. Conditions of severe stringency include higher temperatures of hybridization and washing. The skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as the length of the probe.

Due to the known degeneracy of the genetic code wherein more than one codon can encode the same amino acid, a DNA sequence may vary from that shown in SEQ ID NO:1 and SEQ ID NO:5 and still encode an flt3-L protein having the amino acid sequence of SEQ ID NO:2 and SEQ ID NO:6, respectively. Such variant DNA sequences may result from silent mutations (e.g., occurring during PCR amplification), or may be the product of deliberate mutagenesis of a native sequence.

The invention provides equivalent isolated DNA sequences encoding biologically active flt3-L, selected from: (a) DNA derived from the coding region of a native mammalian flt3-L gene; (b) cDNA comprising the nucleotide sequence presented in SEQ ID NO:1 or SEQ ID NO:5; (c) DNA capable of hybridization to a DNA of (a) under moderately stringent conditions and which encodes biologically active flt3-L; and (d) DNA which is degenerate as a result of the genetic code to a DNA defined in (a), (b) or (c) and which encodes biologically active flt3-L. Flt3-L proteins encoded by such DNA equivalent sequences are encompassed by the invention.

DNA that are equivalents to the DNA sequence of SEQ ID NO:1 or SEQ ID NO:5, will hybridize under moderately stringent conditions to the native DNA sequence that encode polypeptides comprising amino acid sequences of 28-163 of SEQ ID NO:2 or 28-160 of SEQ ID NO:6. Examples of flt3-L proteins encoded by such DNA, include, but are not limited to, flt3-L fragments (soluble or membrane-bound) and flt3-L proteins comprising inactivated N-glycosylation site(s), inactivated KEX2 protease processing site(s), or conservative amino acid substitution(s), as described above. Flt3-L proteins encoded by DNA derived from other mammalian species, wherein the

useful for purifying, screening or separating such flt3-expressing cells from solution. Methods of releasing positively selected cells from the solid phase are known in the art and encompass, for example, the use of enzymes. Such enzymes are preferably non-toxic and non-injurious to the cells and are preferably directed to cleaving the cell-surface binding partner. In the case of flt3:flt3-L interactions, the enzyme preferably would cleave the flt3 receptor, thereby freeing the resulting cell suspension from the "foreign" flt3-L material. The purified cell population then may be expanded *ex vivo* prior to transplantation to a patient in an amount sufficient to reconstitute the patient's hematopoietic and immune system.

Alternatively, mixtures of cells suspected of containing flt3$^+$ cells first can be incubated with a biotinylated flt3-binding protein. Incubation periods are typically at least one hour in duration to ensure sufficient binding to flt3. The resulting mixture then is passed through a column packed with avidin-coated beads, whereby the high affinity of biotin for avidin provides the binding of the cell to the beads. Use of avidin-coated beads is known in the art. See Berenson, et al. *J. Cell. Biochem.*, 10D:239 (1986). Wash of unbound material and the release of the bound cells is performed using conventional methods.

In the methods described above, suitable flt3-binding proteins are flt3-L, anti-flt3 antibodies, and other proteins that are capable of high-affinity binding of flt3. A preferred flt3-binding protein is flt3-L.

As described above, flt3-L of the invention can be used to separate cells expressing flt3 receptors. In an alternative method, flt3-L or an extracellular domain or a fragment thereof can be conjugated to a detectable moiety such as ^{125}I to detect flt3 expressing cells. Radiolabeling with ^{125}I can be performed by any of several standard methodologies that yield a functional ^{125}I-flt3-L molecule labeled to high specific activity. Or an iodinated or biotinylated antibody against the flt3 region or the Fc region of the molecule could be used. Another detectable moiety such as an enzyme that can catalyze a colorimetric or fluorometric reaction, biotin or avidin may be used. Cells to be tested for flt3 receptor expression can be contacted with labeled flt3-L. After incubation, unbound labeled flt3-L is removed and binding is measured using the detectable moiety.

The binding characteristics of flt3-L (including variants) may also be determined using the conjugated, soluble flt3 receptors (for example, ^{125}I-flt3:Fc) in competition

operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are "operably linked" when the regulatory sequence functionally relates to the flt3-L DNA sequence. Thus, a promoter nucleotide sequence is operably linked to a flt3-L DNA sequence if the promoter nucleotide sequence controls the transcription of the flt3-L DNA sequence. The ability to replicate in the desired host cells, usually conferred by an origin of replication, and a selection gene by which transformants are identified, may additionally be incorporated into the expression vector.

In addition, sequences encoding appropriate signal peptides that are not naturally associated with flt3-L can be incorporated into expression vectors. For example, a DNA sequence for a signal peptide (secretory leader) may be fused in-frame to the flt3-L sequence so that flt3-L is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells enhances extracellular secretion of the flt3-L polypeptide. The signal peptide may be cleaved from the flt3-L polypeptide upon secretion of flt3-L from the cell.

Suitable host cells for expression of flt3-L polypeptides include prokaryotes, yeast or higher eukaryotic cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual,* Elsevier, New York, (1985). Cell-free translation systems could also be employed to produce flt3-L polypeptides using RNAs derived from DNA constructs disclosed herein.

Prokaryotes include gram negative or gram positive organisms, for example, *E. coli* or *Bacilli*. Suitable prokaryotic host cells for transformation include, for example, *E. coli, Bacillus subtilis, Salmonella typhimurium,* and various other species within the genera *Pseudomonas, Streptomyces,* and *Staphylococcus*. In a prokaryotic host cell, such as *E. coli*, a flt3-L polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant flt3-L polypeptide.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning

be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) into the above-described yeast vectors.

The yeast α-factor leader sequence may be employed to direct secretion of the flt3-L polypeptide. The α-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. See, e.g., Kurjan et al., *Cell* 30:933, 1982; Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984; U. S. Patent 4,546,082; and EP 324,274. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929, 1978. The Hinnen et al. protocol selects for Trp$^+$ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

Yeast host cells transformed by vectors containing ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems could also be employed to express recombinant flt3-L polypeptides. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988). Established cell lines of mammalian origin also may be employed. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., *Cell* 23:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines, and the CV-1/EBNA-1 cell line derived from the African green monkey kidney cell line CVI (ATCC CCL 70) as described by McMahan et al. (*EMBO J.* 10: 2821, 1991).

Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. Commonly used promoter

is known to the skilled artisan, procedures for purifying a recombinant protein will vary according to such factors as the type of host cells employed and whether or not the recombinant protein is secreted into the culture medium.

For example, when expression systems that secrete the recombinant protein are employed, the culture medium first may be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, (e.g., silica gel having pendant methyl or other aliphatic groups) can be employed to further purify flt3-L. Some or all of the foregoing purification steps, in various combinations, are well known and can be employed to provide a substantially homogeneous recombinant protein.

It is possible to utilize an affinity column comprising the ligand binding domain of flt3 receptors to affinity-purify expressed flt3-L polypeptides. Flt3-L polypeptides can be removed from an affinity column using conventional techniques, e.g., in a high salt elution buffer and then dialyzed into a lower salt buffer for use or by changing pH or other components depending on the affinity matrix utilized. Alternatively, the affinity column may comprise an antibody that binds flt3-L. Example 6 describes a procedure for employing flt3-L of the invention to generate monoclonal antibodies directed against flt3-L.

Recombinant protein produced in bacterial culture is usually isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant fluid if a soluble polypeptide, followed by one or more concentration, salting-out, ion exchange, affinity purification or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, $CaPO_4$-mediated DNA transfection, electroporation, or by using gene transfer vectors such as Epstein-Barr virus. Antisense or sense oligonucleotides are preferably introduced into a cell containing the target nucleic acid sequence by insertion of the antisense or sense oligonucleotide into a suitable retroviral vector, then contacting the cell with the retrovirus vector containing the inserted sequence, either *in vivo* or *ex vivo*. Suitable retroviral vectors include, but are not limited to, the murine retrovirus M-MuLV, N2 (a retrovirus derived from M-MuLV), or or the double copy vectors designated DCT5A, DCT5B and DCT5C (see PCT Application US 90/02656).

Sense or antisense oligonucleotides also may be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

Alternatively, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase.

Flt3-L polypeptides of the invention can be formulated according to known methods used to prepare pharmaceutically useful compositions. Flt3-L can be combined in admixture, either as the sole active material or with other known active materials, with pharmaceutically suitable diluents (e.g., Tris-HCl, acetate, phosphate), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed. 1980, Mack Publishing Co. In addition, such compositions can contain flt3-L complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo*

Prior to fusing the murine flt3 cDNA to the N-terminus of cDNA encoding the Fc portion of a human IgG1 molecule, the amplified mouse flt3 cDNA fragment was inserted into Asp718-*Not*I site of pCAV/NOT, described in PCT Application WO 90/05183. DNA encoding a single chain polypeptide comprising the Fc region of a human IgG1 antibody was cloned into the *Spe*I site of the pBLUESCRIPT SK® vector, which is commercially available from Stratagene Cloning Systems, La Jolla, California. This plasmid vector is replicable in *E. coli* and contains a polylinker segment that includes 21 unique restriction sites. A unique *Bgl*II site was introduced near the 5' end of the inserted Fc encoding sequence, such that the *Bgl*II site encompasses the codons for amino acids three and four of the Fc polypeptide.

The encoded Fc polypeptide extends from the N-terminal hinge region to the native C-terminus, i.e., is an essentially full-length antibody Fc region. Fragments of Fc regions, e.g., those that are truncated at the C-terminal end, also may be employed. The fragments preferably contain multiple cysteine residues (at least the cysteine residues in the hinge reaction) to permit interchain disulfide bonds to form between the Fc polypeptide portions of two separate flt3:Fc fusion proteins, forming dimers as discussed above.

An Asp718 restriction endonuclease cleavage site was introduced upstream of the flt3 coding region. An Asp 718-*Not*I fragment of mouse flt3 cDNA (comprising the entire extracellular domain, the transmembrane region, and a small portion of the cytoplasmic domain) was isolated. The above-described Asp718-*Not*I flt3 partial cDNA was cloned into the pBLUESCRIPT SK® vector containing the Fc cDNA, such that the flt3 cDNA is positioned upstream of the Fc cDNA. Single stranded DNA derived from the resulting gene fusion was mutagenized by the method described in Kunkel (*Proc. Natl. Acad. Sci. USA* 82:488, 1985) and Kunkel et al. (*Methods in Enzymol.* 154:367, 1987) in order to perfectly fuse the entire extracellular domain of flt3 to the Fc sequence. The mutagenized DNA was sequenced to confirm that the proper nucleotides had been removed (i.e., transmembrane region and partial cytoplasmic domain DNA was deleted) and that the flt3 and Fc sequences were in the same reading frame. The fusion cDNA was then excised and inserted into a mammalian expression vector designated sfHAV-EO 409 which was cut with *Sal*I-*Not*I, and the *Sal*I and Asp718 ends blunted. The sfHAV-EO vector (also known as pDC406) is described by McMahan et al. (*EMBO J.*, 10; No. 10: 2821-2832 (1991)).

continuous culture for more than 1 year by passage once a week in medium containing 15 ng/ml rHuIL-7. The parent cell line was derived from lymph node cells of SJL/J mice immunized with 50 nmoles $PLP_{139-151}$ peptide and 100 µg *Mycobacterium tuberculosis* H37Ra in Incomplete Freund's Adjuvant. PLP is the proteolipid protein component of the myelin sheath of the central nervous system. The peptide composed of amino acids 139-151 has previously been shown to be the encephalogenic peptide in experimental autoimmune encephalomyelitis (EAE), a murine model for multiple sclerosis in SJL/J mice. (Touhy, V.K., Z. Lu, R.A. Sobel, R.A. Laursen and M.B. Lees; 1989. Identification of an encephalitogenic determinant of myelin proteolipid protein for SJL mice. *J. Immunol.* 142:1523.) After the initial culture in the presence of antigen, the parent cell line, designated PLP7, had been in continuous culture with rHuIL-7 (and without antigen) for more than 6 months prior to cloning.

P7B-0.3A4 proliferates only in response to very high concentrations of $PLP_{139-151}$ peptide in the presence of irradiated syngeneic splenocytes and is not encephalogenic or alloresponsive. This clone proliferates in response to immobilized anti-CD3 MAb, IL-2, and IL-7, but not IL-4.

Binding of flt3:Fc was observed on murine T-cells and human T-cells, and therefore a murine T-cell line was chosen (0.3A4) due to its ease of growth. A murine 0.3A4 cDNA library in sfHAV-EO was prepared as described in McMahan et al. (*EMBO J.*, 10; No:10; 2821-2832 1991). sfHAV-EO is a mammalian expression vector that also replicates in *E. coli*. sfHAV-EO contains origins of replication derived from SV40, Epstein-Barr virus and pBR322 and is a derivative of HAV-EO described by Dower et al., *J Immunol.* 142:4314 (1989). sfHAV-EO differs from HAV-EO by the deletion of the intron present in the adenovirus 2 tripartite leader sequence in HAV-EO. Briefly, murine T-cell cDNA was cloned into the *Sal*I site of sfHAV-EO by an adaptor method similar to that described by Haymerle et al (*Nucl. Acids Res.* 14:8615, 1986), using the following oligonucleotide adapter pair:

5' TCGACTGGAACGAGACGACCTGCT 3' SEQ ID NO:3
3' GACCTTGCTCTGCTGGACGA 5' SEQ ID NO:4

Double-stranded, blunt-ended, random-primed cDNA was prepared from 0.3A4 poly (A)+ RNA essentially as described by Gubler and Hoffman, *Gene*, 25:263-269 (1983), using a Pharmacia DNA kit. The above adapters were added to the cDNA as described by Haymerle et al.. Low molecular weight material was removed by passage over

Transfected monolayers of CV-1/EBNA-1 cells were assayed for expression of flt3-L by slide autoradiography essentially as described by Gearing et al. (*EMBO J.* 8:3667, 1989). Transfected CV-1/EBNA-1 cells (adhered to chambered slides) were washed once with binding medium with nonfat dry milk (BM-NFDM) (RPMI medium 1640 containing 25 mg/ml bovine serum albumin (BSA), 2 mg/ml sodium azide, 20 mM HEPES, pH 7.2, and 50 mg/ml nonfat dry milk). Cells were then incubated with flt3:Fc in BM-NFDM (1 µg/ml) for 1 hour at room temperature. After incubation, the cell monolayers in the chambered slides were washed three times with BM-NFDM to remove unbound flt3:Fc fusion protein and then incubated with 40 ng/ml ^{125}I-mouse anti-human Fc antibody (described below) (a 1:50 dilution) for 1 hour at room temperature. The cells were washed three times with BM-NFDM, followed by 2 washes with phosphate-buffered saline (PBS) to remove unbound ^{125}I-mouse anti-human Fc antibody. The cells were fixed by incubating for 30 minutes at room temperature in 2.5% glutaraldehyde in PBS, pH 7.3, washed twice in PBS and air dried. The chamber slides containing the cells were exposed on a Phophorimager (Molecular Dynamics) overnight, then dipped in Kodak GTNB-2 photographic emulsion (6x dilution in water) and exposed in the dark for 3-5 days at 4 °C in a light proof box. The slides were then developed for approximately 4 minutes in Kodak D19 developer (40 g/500 ml water), rinsed in water and fixed in Agfa G433C fixer. The slides were individually examined with a microscope at 25-40x magnification and positive cells expressing flt3-L were identified by the presence of autoradiographic silver grains against a light background.

The mouse anti-human Fc antibody was obtained from Jackson Laboratories. This antibody showed minimal binding to Fc proteins bound to the Fcγ receptor. The antibody was labeled using the Chloramine T method. Briefly, a Sephadex G-25 column was prepared according to the manufacturer's instructions. The column was pretreated with 10 column volumes of PBS containing 1% bovine serum albumin to reduce nonspecific adsorption of antibody to the column and resin. Nonbound bovine serum albumin was then washed from the column with 5 volumes of PBS lacking bovine serum albumin. In a microfuge tube 10 µg of antibody (dissolved in 10 µl of PBS) was added to 50 µl of 50 mM sodium phosphate buffer (pH 7.2) 2.0 mCi of carrier-free Na^{125}I was added and the solution was mixed well. 15 µl of a freshly prepared solution of chloramine-T (2 mg/ml in 0.1 M sodium phosphate buffer (pH 7.2)) was then added and the mixture was incubated for 30 minutes at room temperature, and the mixture then was immediately applied to the column of Sephadex G-25. The radiolabelled antibody was then eluted from the column by collecting 100-

EXAMPLE 4

Cloning of cDNA Encoding Human Flt3-L

A cDNA encoding human flt3-L was cloned from a human clone 22 T cell λgt10 random primed cDNA library as described by Sims et al., *PNAS*, 86:8946-8950 (1989). The library was screened with a 413 bp Ple I fragment corresponding to the extracellular domain of the murine flt3-L (nucleotides 103-516 of SEQ ID NO:1). The fragment was random primed, hybridized overnight to the library filters at 55°C in oligo prehybridization buffer. The fragment was then washed at 55°C at 2 x SSC/0.1% SDS for one hour, followed by 1 x SSC/0.1% SDS for one hour and then by 0.5 x SSC/0,1% SDS for one hour. The DNA from the positive phage plaques was extracted, and the inserts were amplified by PCR using oligonucleotides specific for the phage arms. The DNA then was sequenced, and the sequence for clone #9 is shown in SEQ ID NO:5. Additional human flt3-L cDNA was isolated from the same λgt10 random primed cDNA library as described above by screening the library with a fragment of the extracellular domain of the murine clone #5H cDNA comprising a cDNA sequence essentially corresponding to nucleotides 128-541 of SEQ ID NO:1.

Sequencing of the 988 bp cDNA clone #9 revealed an open reading frame of 705 bp surrounded by 29 bp of 5' non-coding sequence and 250 bp of 3' non-coding sequence. The 3' non-coding region did not contain a poly-A tail. There were no in-frame stop codons upstream of the initiator methionine. The open reading frame encodes a type I transmembrane protein of 235 amino acids as shown by amino acids 1-235 of SEQ ID NO:6. The protein has an N-terminal signal peptide of alternatively 26 or 27 amino acids. There exists a slightly greater probability that the N-terminal signal peptide is 26 amino acids in length than 27 amino acids in length. The signal peptide is followed by a 156 or a 155 amino acid extracellular domain (for signal peptides of 26 and 27 amino acids, respectively); a 23 amino acid transmembrane domain and a 30 amino acid cytoplasmic domain. Human flt3-L shares overall 72% amino acid identity and 78% amino acid similarity with murine flt3-L. The vector pBLUESCRIPT SK(-) containing the human flt3-L cDNA of clone #9 was deposited with the American Type Culture Collection, Rockville, Maryland, USA (ATCC) on August 6, 1993 and assigned acession number ATCC 69382. The deposit was made under the terms of the Budapest Treaty.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of flt3-L in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line, e.g., NS1 or preferably P3x63Ag8.653 (ATCC CRL 1580). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified flt3-L by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971 and in U.S. Patent 4,703,004. A preferred screening technique is the antibody capture technique described in Beckmann et al., (*J. Immunol.* 144:4212, 1990) Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-flt3-L-L monoclonal antibodies. Alternatively, hybridoma cells can be grown *in vitro* in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can also be used, as can affinity chromatography based upon binding to flt3 L.

EXAMPLE 7

Use of Flt3-L Alone and in Combination with IL-7 or IL-3

This example demonstrates the stimulation and proliferation of $AA4.1^+$ fetal liver cells by compositions containing flt3-L and IL-7; as well as the stimulation and proliferation of c-*kit*-positive (c-kit^+) cells by compositions containing flt3-L and IL-3.

AA4.1-positive ($AA4.1^+$) expressing cells were isolated from the livers of day 14 fetal C57BL/6 mice by cell panning in Optilux 100 mm plastic Petri dishes (Falcon No. 1001, Oxnard, CA). Plates were coated overnight at 4 °C in PBS plus 0.1% fetal bovine serum (FBS) containing 10 µg/ml AA4.1 antibody (McKearn et. al., *J. Immunol.*, 132:332-339, 1984) and then washed extensively with PBS plus 1% FBS prior to use. A single cell suspension of liver cells was added at 10^7 cells/dish in PBS plus 1% FBS and allowed to adhere to the plates for two hours at 4 °C. The plates were then extensively washed, and the adhering cells were harvested by scraping for

TABLE I

Effect of Flt3-L and IL-7 on Proliferation of AA4.1+ Fetal Liver Cells.

	Factor			
	Control	flt3-L	IL-7	flt3-L + IL-7
[^{3}H]-thymidine incorporation (CPM)	100	1000	100	4200

The combination of flt3-L and IL-7 produced a response that was approximately four-fold greater than flt3-L alone and approximately 40-fold greater than IL-7 alone.

TABLE II

Effect of Flt3-L and IL-3 on Proliferation of C-*kit*+ Cells.

	Factor			
	Control (vector alone)	flt3-L	IL-3	flt3-L + IL-3
[^{3}H]-thymidine incorporation (CPM)	100	1800	3000	9100

Culture supernatant from CV1/EBNA cells transfected with flt3-L cDNA stimulated the proliferation of c-*kit*$^+$ stem cells approximately 18-fold greater than the culture supernatant of CV1/EBNA cells transfected with the expression vector alone. Addition of IL-3 to flt3-L containing supernatant showed a synergistic effect, with approximately twice the degree of proliferation observed than would be expected if the effects were additive.

EXAMPLE 8

Construction of Flt3-L:Fc Fusion Protein

This example describes a methof for constructing a fusion protein comprising an extracellular region of the flt3-L and the Fc domain of a human immunoglobulin. The methods are essentially the same as those described in Example 1 for construction of a flt3:Fc fusion protein.

Prior to fusing a flt3-L cDNA to the N-terminus of cDNA encoding the Fc portion of a human IgG1 molecule, the flt3-L cDNA fragment is inserted into Asp718-

the culture medium *via* the flt3-L signal peptide. The fusion protein can be purified on protein A Sepharose columns.

EXAMPLE 9

Generation of Transgenic Mice That Overexpress Flt3-L

This example describes a procedure used to generate transgenic mice that overexpress flt3-L. Flt3-L-overexpressing transgenic mice were studied to determine the biological effects of overexpression. Mouse (B16/J) pronuclei were microinjected with flt3-L DNA according to the method described by Gordon et al., *Science* 214:1244-1246, (1981). In general, fertilized mouse eggs having visible pronuclei were first placed on an injection chamber and held in place with a small pipet. An injection pipet was then used to inject the gene encoding the flt3-L (clone #6C) into the pronuclei of the egg. Injected eggs were then either (i) transferred into the oviduct of a 0.5 day p.c. pseudopregnant female; (ii) cultured *in vitro* to the two-cell stage (overnight) and transferred into the oviduct of a 0.5 day p.c. pseudopregnant female; or (iii) cultured *in vitro* to the blastocyst stage and transferred into the uterus of a 2.5 day p.c. pseudopregnant female. Preferably, either of the first two options can be used since they avoid extended *in vitro* culture, and preferably, approximately 20-30 microinjected eggs should be transferred to avoid small litters.

EXAMPLE 10

Flt3-L Stimulates Proliferation of Erythroid Cells in the Spleen

This example describes the effect of flt3-L on the production of erythroid cells in the spleen of transgenic mice. Transgenic mice were generated according to the procedures of Example 10. The mice were sacrificed and each intact spleen was made into a single cell suspension. The suspended cells were spun and then resuspended in 10 ml of medium that contained PBS + 1% fetal bovine serum. Cell counts were performed thereon using a hemocytometer. Each cell specimen was counted with Trypan Blue stain to obtain a total viable cell count per milliliter of medium according to the following formula: (RBC + WBC)/ ml, wherein RBC is the red blood cell count and WBC means the white blood cell count. Each specimen then was counted with Turk's stain to obtain a total white blood cell count per milliliter of medium. The total red blood cell count per milliliter was calculated for each specimen by subtracting the total white blood cell count per milliliter from the total viable cell count per milliliter. The results are shown in the following Table III.

TABLE IV

Effect of flt3-L Overexpression in Transgenic Mice

	Percentage of Positive Cells			
Marker	Unrelated Control	Littermate Control	Transgenic #1	Transgenic #2
B220	30.64	27.17	45.84	48.78
sIgM	3.54	2.41	1.94	1.14
S7(CD43)	54.43	45.44	46.11	50.59
SCA-1	10.92	11.74	19.45	27.37
CD4	6.94	8.72	12.21	14.05
Mac-1	36.80	27.15	21.39	18.63

The above data indicate that flt3-L overexpression in mice leads to an increase in the number of B cells, as indicated by the increase $B220^+$ cells and $SCA\text{-}1^+$ cells. Analysis of $B220^+$ cells by FACS indicated an increase in proB cells (HSA^-, $S7^+$). The increase in $CD4^+$ cells indicated an approximate two-fold increase in T cells and stem cells. The decrease in cells having the sIgM marker indicated that flt3-L does not stimulate proliferation of mature B cells. These data indicate that flt3-L increases cells with a stem cell, T cell or an early B cell phenotype, and does not stimulate proliferation of mature B cells or macrophages.

EXAMPLE 12

Analysis of the Thymus From Flt3-L-Over-expressing Mice

This Example describes the analysis of the thymus from the transgenic mice generated according to the procedure of Example 10. Six adult mice, each approximately three months of age, were sacrificed. The thymus from each mouse was removed and a single cell suspension was made.

FACS analysis demonstrated that no total change in cell number occurred and that the mice showed no change in the ratios of maturing thymocytes using the markers: CD4 vs. CD8; CD3 vs. αßTCR (T cell receptor); and CD3 vs. γδTCR (T cell receptor). However, a change in the ratios of certain cell types within the $CD4^-$ and $CD8^-$ compartment (i.e., the earliest cells with respect to development; which represent approximately 2% to 3% of total thymus cells) occurred. Specifically, $CD4^-$ and $CD8^-$ cells in the thymus develop in three stages. Stage 1 represents cells having the $Pgp\text{-}1^{++}$, HSA^+ and IL-2 receptor-negative ("$IL\text{-}2R^-$") markers. After stage 1, thymic cells develop to stage 2 consisting of cells having $Pgp\text{-}1^+$, HSA^{++}, and $IL\text{-}2R^{++}$ markers, and then to stage 3, characterized by cells having $Pgp\text{-}1^{+/-}$, HSA^{++}, and $IL\text{-}2R^-$

stain, and CFU-GM colonies are scored using a dissecting microscope (Ward et al., *Exp. Hematol.*, 16:358 (1988). Alternatively, CFU-GM colonies can be assayed using the CD34/CD33 flow cytometry method of Siena et al., *Blood*, Vol. 77, No. 2, pp 400-409 (1991), or any other method known in the art.

CFU-GM containing cultures are frozen in a controlled rate freezer (e.g., Cryo-Med, Mt. Clemens, MI), then stored in the vapor phase of liquid nitrogen. Ten percent dimethylsulfoxide can be used as a cryoprotectant. After all collections from the patient have been made, CFU-GM containing cultures are thawed and pooled. The thawed cell collection either is reinfused intravenoulsy to the patient or expanded *ex vivo* prior to reinfusion. *Ex vivo* expansion of pooled cells can be performed using flt3-L as a growth factor either alone, sequentially or in concurrent combination with other cytokines listed above. Methods of such *ex vivo* expansion are well known in the art. The cells, either expanded or unexpanded, are reinfused intravenously to the patient. To facilitate engraftment of the transplanted cells, flt3-L is administered simultaneously with, or subsequent to, the reinfusion. Such administration of flt3-L is made alone, sequentially or in concurrent combination with other cytokines selected from the list above.

EXAMPLE 14

Purification of Hematopoietic Progenitor and Stem Cells Using Flt3-L

This Example describes a method for purifying hematopoietic progenitor cells and stem cells from a suspension containing a mixture of cells. Cells from bone marrow and peripheral blood are collected using conventional procedures. The cells are suspended in standard media and then centrifuged to remove red blood cells and neutrophils. Cells located at the interface between the two phases (also known in the art as the buffy coat) are withdrawn and resuspended. These cells are predominantly mononuclear and represent a substantial portion of the early hematopoietic progenitor and stem cells. The resulting cell suspension then is incubated with biotinylated flt3-L for a sufficient time to allow substantial flt3:flt3-L interaction. Typically, incubation times of at least one hour are sufficient. After incubation, the cell suspension is passed, under the force of gravity, through a column packed with avidin-coated beads. Such columns are well known in the art, see Berenson, et al., *J. Cell Biochem.*, 10D:239 (1986). The column is washed with a PBS solution to remove unbound material. Target cells can be released from the beads and from flt3-L using conventional methods.

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 879 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..25

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 855..879

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 57..752

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
GTCGACTGGA ACGAGACGAC CTGCTCTGTC ACAGGCATGA GGGGTCCCCG GCAGAG      56

ATG ACA GTG CTG GCG CCA GCC TGG AGC CCA AAT TCC TCC CTG TTG CTG   104
Met Thr Val Leu Ala Pro Ala Trp Ser Pro Asn Ser Ser Leu Leu Leu
 1               5                  10                  15

CTG TTG CTG CTG CTG AGT CCT TGC CTG CGG GGG ACA CCT GAC TGT TAC   152
Leu Leu Leu Leu Leu Ser Pro Cys Leu Arg Gly Thr Pro Asp Cys Tyr
            20                  25                  30
TTC AGC CAC AGT CCC ATC TCC TCC AAC TTC AAA GTG AAG TTT AGA GAG   200
Phe Ser His Ser Pro Ile Ser Ser Asn Phe Lys Val Lys Phe Arg Glu
        35                  40                  45

TTG ACT GAC CAC CTG CTT AAA GAT TAC CCA GTC ACT GTG GCC GTC AAT   248
Leu Thr Asp His Leu Leu Lys Asp Tyr Pro Val Thr Val Ala Val Asn
    50                  55                  60

CTT CAG GAC GAG AAG CAC TGC AAG GCC TTG TGG AGC CTC TTC CTA GCC   296
Leu Gln Asp Glu Lys His Cys Lys Ala Leu Trp Ser Leu Phe Leu Ala
65                  70                  75                  80

CAG CGC TGG ATA GAG CAA CTG AAG ACT GTG GCA GGG TCT AAG ATG CAA   344
Gln Arg Trp Ile Glu Gln Leu Lys Thr Val Ala Gly Ser Lys Met Gln
                85                  90                  95

ACG CTT CTG GAG GAC GTC AAC ACC GAG ATA CAT TTT GTC ACC TCA TGT   392
Thr Leu Leu Glu Asp Val Asn Thr Glu Ile His Phe Val Thr Ser Cys
            100                 105                 110
```

```
Leu Gln Asp Glu Lys His Cys Lys Ala Leu Trp Ser Leu Phe Leu Ala
65                  70                  75                  80

Gln Arg Trp Ile Glu Gln Leu Lys Thr Val Ala Gly Ser Lys Met Gln
                85                  90                  95

Thr Leu Leu Glu Asp Val Asn Thr Glu Ile His Phe Val Thr Ser Cys
            100                 105                 110

Thr Phe Gln Pro Leu Pro Glu Cys Leu Arg Phe Val Gln Thr Asn Ile
        115                 120                 125

Ser His Leu Leu Lys Asp Thr Cys Thr Gln Leu Leu Ala Leu Lys Pro
    130                 135                 140

Cys Ile Gly Lys Ala Cys Gln Asn Phe Ser Arg Cys Leu Glu Val Gln
145                 150                 155                 160

Cys Gln Pro Asp Ser Ser Thr Leu Leu Pro Pro Arg Ser Pro Ile Ala
                165                 170                 175

Leu Glu Ala Thr Glu Leu Pro Glu Pro Arg Pro Arg Gln Leu Leu Leu
            180                 185                 190

Leu Leu Leu Leu Leu Pro Leu Thr Leu Val Leu Leu Ala Ala Ala Trp
        195                 200                 205

Gly Leu Arg Trp Gln Arg Ala Arg Arg Arg Gly Glu Leu His Pro Gly
    210                 215                 220

Val Pro Leu Pro Ser His Pro
225                 230
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCGACTGGAA CGAGACGACC TGCT 24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

```
CGC TTC GTC CAG ACC AAC ATC TCC CGC CTC CTG CAG GAG ACC TCC GAG   437
Arg Phe Val Gln Thr Asn Ile Ser Arg Leu Leu Gln Glu Thr Ser Glu
                125                 130                 135

CAG CTG GTG GCG CTG AAG CCC TGG ATC ACT CGC CAG AAC TTC TCC CGG   485
Gln Leu Val Ala Leu Lys Pro Trp Ile Thr Arg Gln Asn Phe Ser Arg
            140                 145                 150

TGC CTG GAG CTG CAG TGT CAG CCC GAC TCC TCA ACC CTG CCA CCC CCA   533
Cys Leu Glu Leu Gln Cys Gln Pro Asp Ser Ser Thr Leu Pro Pro Pro
        155                 160                 165

TGG AGT CCC CGG CCC CTG GAG GCC ACA GCC CCG ACA GCC CCG CAG CCC   581
Trp Ser Pro Arg Pro Leu Glu Ala Thr Ala Pro Thr Ala Pro Gln Pro
    170                 175                 180

CCT CTG CTC CTC CTA CTG CTG CTG CCC GTG GGC CTC CTG CTG CTG GCC   629
Pro Leu Leu Leu Leu Leu Leu Leu Pro Val Gly Leu Leu Leu Leu Ala
185                 190                 195                 200

GCT GCC TGG TGC CTG CAC TGG CAG AGG ACG CGG CGG AGG ACA CCC CGC   677
Ala Ala Trp Cys Leu His Trp Gln Arg Thr Arg Arg Arg Thr Pro Arg
                205                 210                 215

CCT GGG GAG CAG GTG CCC CCC GTC CCC AGT CCC CAG GAC CTG CTG CTT   725
Pro Gly Glu Gln Val Pro Pro Val Pro Ser Pro Gln Asp Leu Leu Leu
            220                 225                 230

GTG GAG CAC TGACCTGGCC AAGGCCTCAT CCTGCGGAGC CTTAAACAAC          774
Val Glu His
        235

GCAGTGAGAC AGACATCTAT CATCCCATTT TACAGGGGAG GATACTGAGG CACACAGAGG 834

GGAGTCACCA GCCAGAGGAT GTATAGCCTG GACACAGAGG AAGTTGGCTA GAGGCCGGTC 894

CCTTCCTTGG GCCCCTCTCA TTCCCTCCCC AGAATGGAGG CAACGCCAGA ATCCAGCACC 954

GGCCCCATTT ACCCAACTCT GAACAAAGCC CCCG                             988
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 235 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Thr Val Leu Ala Pro Ala Trp Ser Pro Thr Thr Tyr Leu Leu Leu
 1               5                  10                  15

Leu Leu Leu Leu Ser Ser Gly Leu Ser Gly Thr Gln Asp Cys Ser Phe
            20                  25                  30

Gln His Ser Pro Ile Ser Ser Asp Phe Ala Val Lys Ile Arg Glu Leu
        35                  40                  45
```

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATATGGATCC CTACTGCCTG GGCCGAGGCT CTGGGAG 37

19. A host cell transformed or transfected with the expression vector according to claim 14.
20. A host cell transformed or transfected with the expression vector according to claim 15.
21. A host cell transformed or transfected with the expression vector according to claim 16.
22. A host cell transformed or transfected with the expression vector according to claim 17.
23. A process for producing a flt3-L polypeptide, comprising culturing a host cell according to claim 18 under conditions promoting expression, and recovering the polypeptide from the culture medium.
24. A process for producing a flt3-L polypeptide, comprising culturing a host cell according to claim 19 under conditions promoting expression, and recovering the polypeptide from the culture medium.
25. A process for producing a flt3-L polypeptide, comprising culturing a host cell according to claim 20 under conditions promoting expression, and recovering the polypeptide from the culture medium.
26. A process for producing a flt3-L polypeptide, comprising culturing a host cell according to claim 21 under conditions promoting expression, and recovering the polypeptide from the culture medium.
27. A process for producing a flt3-L polypeptide, comprising culturing a host cell according to claim 22 under conditions promoting expression, and recovering the polypeptide from the culture medium.
28. An antibody that is immunoreactive with a flt3-L polypeptide.
29. An antibody according to claim 28 that is a monoclonal antibody.
30. A pharmaceutical composition comprising an effective amount of a flt3-L polypeptide according to claim 1 and a pharmaceutically acceptable carrier, excipient or diluent.
31. A pharmaceutical composition comprising an effective amount of a flt3-L polypeptide according to claim 3 and a pharmaceutically acceptable carrier, excipient or diluent.
32. A pharmaceutical composition comprising an effective amount of a flt3-L polypeptide according to claim 5 and a pharmaceutically acceptable carrier, excipient or diluent.
33. A method for conducting autologous transplantation in a patient receiving cytoreductive therapy, comprising:

40. A method of stimulating the proliferation of cells of the erythroid lineage in the spleen of a mammal comprising administering to the mammal an effective amount of a flt3-L polypeptide according to claim 1.

41. A method according to claim 40, further comprising the administration of an effective amount of EPO.

42. A method of treating a patient having symptoms of myelodysplastic syndrome, comprising the administration to the patient of an effective amount of a flt3-L polypeptide according to claim 1 and, optionally, an effective amount of one or more growth factors selected from the group consisting of CSF-1, GM-CSF, SF, G-CSF, EPO, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF/IL-3 fusion proteins, LIF and FGF.

43. A method of treating a patient having symptoms of anemia, comprising the administration to the patient of an effective amount of a flt3-L polypeptide according to claim 1 and, optionally, an effective amount of one or more growth factors selected from the group consisting of CSF-1, GM-CSF, SF, G-CSF, EPO, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF/IL-3 fusion proteins, LIF and FGF.

44. A method of treating a patient having symptoms of acquired immune deficiency syndrome, comprising the administration to the patient of an effective amount of a flt3-L polypeptide according to claim 1 and, optionally, an effective amount of one or more growth factors selected from the group consisting of CSF-1, GM-CSF, SF, G-CSF, EPO, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF/IL-3 fusion proteins, LIF and FGF.

45. A method according to claim 44, wherein the patient is receiving AZT therapy.

46. A transgenic non-human mammal all of whose germ and somatic cells contain a DNA sequence according to claim 8 introduced into said mammal, or an ancestor of said mammal, at an embryonic stage.

47. A method of separating cells having the flt3 receptor on the surface thereof from a mixture of cells in suspension, comprising contacting the cells in the mixture with a contacting surface having a flt3-binding protein thereon, and separating the contacting surface and the suspension.

48. A method according to claim 47, wherein the flt3-binding protein is flt3-L.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05365

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Cell, Volume 75, issued 17 December 1993, S.D. Lyman et al., "Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells", pages 1157-1167, entire document.	1-32, 39-45, 47, 48
Y	US, A, 5,199,942 (GILLIS) 6 April 1993, see especially Abstract.	33-35, 37, 38
Y	US, A, 5,192,553 (BOYSE ET AL.) 9 March 1993, entire document, especially column 20 and Table 2, columns 27-29.	42-45, 47, 48
Y	US, A, 4,745,099 (AKAMATSU ET AL.) 17 May 1988, column 1 line 33 to column 2, line 26.	43
Y	US, A, 5,013,824 (ABRAMS ET AL.) 7 May 1991, column 2, lines 40-51.	43
Y	US,A, 5,057,420 (MASSEY) 15 October 1991, entire document.	46
Y	US, A, 5,114,710 (TAKAKU ET AL.) 19 May 1992, column 1 lines 40-48.	43
Y	Science, Volume 246, issued 8 December 1989, D. Hanahan, "Transgenic mice as probes into complex systems", pages 1265-1275, entire document.	46

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05365

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

G01N 1/34, 33/48; C12N 5/00, 1/21; A61K 37/02, 48/00; C07K 13/00, 15/28; C07H 21/04; C12N 15/12, 15/64; A01K 67/00

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-27 and 30-32, drawn to flt3 ligand protein, DNA, vectors, transformed cells, and recombinant production of protein.

Group II, claims 28 and 29, drawn to anti flt3-ligand antibodies.

Group III, claims 33-35, drawn to a method for conducting autologous transplantation.

Group IV, claim 36, drawn to cell culture medium.

Group V, claim 37, drawn to a transfection method.

Group VI, claim 38, drawn to a gene therapy method.

Group VII, claims 39-45, drawn to an in vivo method of treatment to cause stem cell proliferation.

Group VIII, claim 46, drawn to transgenic animals.

Group IX, claims 47 and 48, drawn to a cell sorting method.

The inventions listed as Groups I-IX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group II is drawn to a product which is independent and distinct from the products of group I, and which is not linked by a special technical feature within the meaning of PCT Rule 13.2 to such products so as to form a single inventive concept. Groups III-IX are drawn to multiple methods of use of flt3 ligand or nucleic acids encoding such. These methods are independent and distinct and are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept. Note that PCT Rule 13 does not provide for multiple methods within a single general inventive concept.